

Amendments to the Specification

Please delete the section of the application entitled "Sequence Listing" immediately after the section of the specification entitled "Abstract" on page 73 and insert the enclosed Sequence Listing therefor.

Please replace the paragraph beginning at page 5, line 12, with the following redlined paragraph:

--FIG. 4 shows that anti-PY and EPEC 78 kDa antibodies label identical structures in infected mammalian cells. Immunofluorescent labeling of HeLa cells following 3 hr EPEC infection. Fixed and permeabilized cells were co-labeled with either anti-PY and anti-EPEC 78 kDa antibodies (FIG. 4A-4D), or FITC-phalloidin and anti-EPEC 78 kDa (FIG. 4E-4H). Panels FIG. 4D and FIG. 4H are superimpositions of panels FIG. 4B and FIG. 4C, and FIG. 4F and FIG. 4G, respectively. For panels FIG. 4E- FIG. 4H, fields were selected to show infected cells with most developed actin pedestals. Arrows indicate nonadherent bacteria.

Please replace the paragraph beginning at page 5, line 19, with the following redlined paragraph:

--FIG. 5 shows that the transfer of Tir to host cells is dependent on the type III secretion apparatus and the EPEC secreted proteins EspA and EspB. HeLa cells were infected with EPEC or strains containing mutations in *eeA* (intimin), *espA*, *tir*, or *cfm-14* and their Triton® X-100 soluble (membrane) and insoluble (bacteria and cytoskeleton) fractions isolated. Samples were resolved by SDS-6% PAGE and transferred to nitrocellulose prior to probing with anti-EPEC 78 kDa antibodies. Tir related proteins (90, 78 kDa) are indicated by arrows. Molecular mass markers are in kDa.--

Please replace the paragraph beginning at page 5, line 26, with the following redlined paragraph:

--FIG. 6 shows the nucleotide sequence (SEQ ID NO:10) and predicted protein (SEQ ID NO:11) of *tir* (FIG. 6A-B) and genetic map (~~B~~FIG. 6C). In FIG. 6A-B, two putative membrane spanning domains are underlined, and the 6 tyrosine residues are ~~shaded~~shown in boxes. In FIG. ~~6B~~6C, the location of *tir* in Locus of Enterocyte Effacement (LEE) and the gene deletion strategy are diagramed.--

Please replace the paragraph beginning at page 7, line 27, with the following redlined paragraph:

--FIG. 9A-B shows the sequence similarity between Tir polypeptides from EPEC (~~SEQ ID NO:2~~) (SEQ ID NO:10) and EHEC (~~SEQ ID NO:4~~) (SEQ ID NO:11).--

Please replace the paragraph beginning at page 14, line 24, with the following redlined paragraph:

--Tir polypeptides included in the invention can have one of the amino acid sequences of Tir from pathogenic *E. coli*, for example, the amino acid sequence of SEQ ID NO:210 and ~~SEQ ID NO:4~~SEQ ID NO:11.--

Please replace the paragraph beginning at page 16, line 29, with the following redlined paragraph:

--Other Tir polypeptides included in the invention are polypeptides having amino acid sequences that are at least 50% identical to the amino acid sequence of a Tir polypeptide, such as SEQ ID NO:210 and ~~SEQ NO:4~~SEQ ID NO:11. The length of comparison in determining amino acid sequence homology can be, for example, at least 15 amino acids, for example, at least 20,25, or 35 amino acids. Homology can be measured using standard sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705; also see Ausubel, *et al.*, *supra*).--

Please replace the paragraph beginning at page 17, line 25, with the following redlined paragraph:

--The invention also provides isolated polynucleotides that encode the Tir polypeptides described above, as well as fragments thereof. For example, isolated polynucleotides may encode the Tir polypeptides with the amino acid sequences of SEQ ID NO:210 and ~~SEQ ID NO:4~~SEQ ID NO:11. These polynucleotides can contain naturally occurring nucleotide sequences or sequences that differ from those of the naturally occurring nucleic acids that encode Tir, but encode the same amino acids, due to the degeneracy of the genetic code. The nucleic acids of the invention can contain DNA or RNA nucleotides, or combinations or modifications thereof.--

Please replace the paragraph beginning at page 20, line 27, with the following redlined paragraph:

--This invention encompasses nucleic acid molecules that hybridize to the polynucleotide of the invention. As used herein, the term "nucleic acid" encompasses RNA as well as single and double-stranded DNA and cDNA. The polynucleotide encoding Tir includes SEQ ID NO:1 and SEQ ID NO:3, as well as nucleic acid sequences complementary to that sequence. A complementary sequence may include an antisense nucleotide. When the sequence is RNA, the deoxynucleotides A, G, C, and T of SEQ ID NO:1 and SEQ ID NO:3 are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the protein of SEQ ID NO:210 and ~~SEQ ID NO:4~~SEQ ID NO:11 under physiological conditions.--

Please replace the paragraph beginning at page 38, line 5, with the following redlined paragraph:

--Polyclonal mouse and rat antibodies to the 78 kDa EPEC protein were generated as follows: High levels of EPEC protein secretion were induced as above and supernatants concentrated by the addition of 40% ammonium sulfate (wt/vol) overnight. After centrifugation the resulting pellet was resuspended in phosphate-buffered saline (PBS) plus

phenylmethylsulfonyl fluoride (PMSF, 0.1 mM final conc.) and dialyzed against PBS. The concentrated proteins were resuspended in loading buffer and resolved by SDS-12% PAGE. After transferring to nitrocellulose the proteins were visualized by Ponceau red, excised, fragmented by sonication, and used to immunize mice and rats. The titer was assessed by immunoblot analysis against EPEC/CVD450 secreted proteins. Antisera was used at 1:2,000 – 1:5,000 in immunoblot analysis, and at 1:100 – 1:200 for immunofluorescence microscopy. EPEC-infected HeLa cells were fractionated according to the method of Kenny and Finlay, *Infection & Immunity* 65 (1997), using saponin to release cytoplasmic proteins and Triton® X-100 to solubilize membrane proteins and these fractions were probed with both anti-PY (to detect Hp90) and anti-EPEC 78 kDa protein antibodies. HeLa (CCL 2, ATCC) cells were cultured in Dulbecco's Modified Eagles Media containing 10% fetal calf serum.--

Please replace the paragraph beginning at page 39, line 6, with the following redlined paragraph:

--Because high levels of Hp90 are detected in J774 macrophage-like cells (TIB 67, ATCC), J774 cells were infected with the intimin mutant, CVD206, to maximize membrane levels of Hp90, fractionated, and the Triton® X-100 soluble membrane fraction immunoprecipitated with either anti-PY or anti-EPEC 78 kDa antibodies. J774 A.1 cells were cultured in Dulbecco's Modified Eagles Media containing 10% fetal calf serum. Immunoprecipitation was carried out as described by Kenny and Finlay, *Infection & Immunity* 65 (1997). The immunoprecipitates and post-immunoprecipitate supernatants were then resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-PY antibodies (FIG. 2A). Using this procedure, Hp90 was cleared from the membrane fraction of infected cells with both antibodies (supernatant in FIG. 2A) and a co-migrating 90 kDa protein was recognized in the immunoprecipitate with both antibodies.--

Please replace the paragraph beginning at page 39, line 17, with the following redlined paragraph:

--Additional evidence that both antibodies were recognizing the same protein was obtained by performing two dimensional gel electrophoresis. Two dimensional gel electrophoresis was performed as follows: Triton® X-100 soluble membrane fractions from J774 cells were prepared as described by Rosenshine *et al.*, *EMBO J.* 15:2613-2624 (1996). The membranes were further purified by heating at 90°C for 5 min followed by ultracentrifugation at 50,000 x g, 30 min, 4°C. 100 µl solubilized membrane protein was precipitated using CHCl₃/acetone/H₂O, resuspended in 12.5 µl 2D gel sample buffer, and resolved on first-dimension isoelectric focusing gels, using 4% (w/v) polyacrylamide mini-isoelectric focusing gels (BioRad) containing 2% ampholines (0.4% pH 3-10, 1.6% pH 5-7) at 700 V, 3.5 hrs. Second dimension electrophoresis was performed on 8% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose for immunoblotting with the appropriate antisera. Membranes containing Hp90 were isolated from CVD206 infected J774, and proteins separated by isoelectric focusing followed by SDS-PAGE. Duplicate samples were transferred to nitrocellulose and probed with either anti-PY or anti-EPEC 78 kDa antibodies (FIG. 2B).--

Please replace the paragraph beginning at page 40, line 10, with the following redlined paragraph:

--Tir in host cell membranes has a significantly different predicated molecular mass than the protein secreted from bacteria (90 kDa versus 78 kDa on SDS-PAGE). The difference was due to tyrosine phosphorylation of the 78 kDa protein in the host cell, which is not recognized by the PY antibodies in its bacterial secreted 78 kDa form (FIG. 3). To show this, membrane extracts prepared from the intimin mutant CVD206 infected HeLa cells were treated with alkaline phosphatase, which should remove all phosphate groups. An immunoblot was performed with the treated sample, probing with anti-EPEC 78 kDa and anti-PY antibodies. Cellular fractionation and alkaline phosphatase treatment was carried out as described by Kenny and Finlay, *Infection & Immunity* 65 (1997). Briefly, cultured HeLa cells were infected with EPEC, washed, and treated with 0.2% saponin to release the soluble cytoplasmic fraction in the

presence of phosphatase and protease inhibitors. One percent Triton® X-100 was used to solubilize the membrane proteins from the remaining insoluble fraction which contains adherent bacteria, host nuclei and cytoskeleton. For alkaline phosphatase treatment, membrane fractions were isolated in the absence of phosphatase inhibitors, and incubated with 2 U of alkaline phosphatase (NEB) for 4 hr at 37°C.--

Please replace the paragraph beginning at page 44, line 1, with the following redlined paragraph:

--Although the Tir protein is predicted to encode a 56.8 kDa protein, a molecular mass of about 78 kDa was observed for the secreted protein, which may reflect some additional bacterial modification or abnormal migration due to amino acid composition or structural features. Tir contains two predicted membrane spanning sequences with six tyrosine residues in the C-terminal half of the protein which may serve as substrates for phosphorylation (FIG. 6A-6B). Tir is predicted to have two transmembrane domains (TM predict, ISREC, Switzerland) with the six tyrosine residues, potential kinase substrates, in the C-terminal half. As predicted for EspA and EspB, Tir appears to be slightly acidic (predicted pI of 5.16) which was verified by two dimensional gel electrophoresis analysis.—

Please replace the paragraph beginning at page 44, line 10, with the following redlined paragraph:

--To show the role of this protein in pedestal formation a chromosomal deletion in the *tir* gene was constructed (FIG. 6B6C). The chromosomal deletion mutant was constructed as follows: the primers MS102 + (5'-AAAGTCGACAAGAACCTGAGAACCAG-3'; SEQ ID NO:8) and MS103 - (5'-TTTGTGCGACTTATGTTTGTGAAGGTAGTGG3'; SEQ ID NO:9) were used to create a 5' deletion of 795 bp between bp 149 and bp 795 of the *tir* gene using inverse PCR amplification of pSK-*tir*. The oligonucleotide MS103 also introduced a *SalI* restriction site into the PCR product and a stop codon to terminate protein translation. The resulting 3000 bp *SalI/SacI tir* deletion fragment was cloned into the positive-selection suicide vector pCVD442 (*SalI/SacI*) and used to construct the deletion mutant by allelic exchange.--

Please replace the paragraph beginning at page 46, line 6, with the following redlined paragraph:

--Gel overlay experiments were performed using EPEC supernatants grown under conditions which express Tir. Gel overlays were performed as follows: Samples were resolved by 12% SDS-PAGE and transferred to nitrocellulose prior to blocking in 5% non-fat milk in Hyb75 (20 mM HEPES [pH 7.7]; 75 mM KCl; 0.1mM EDTA; 2.5 mM MgCl₂; 1 mM DTT; 0.05% NP40) for 2 hr at room temperature. His-T7Intimin was incubated overnight in 1% BSA/TBS (Tris-buffered saline), washed and bound fusion detected by T7 antibodies (1:5000 in 1% BSA/TBS) followed by goat anti-mouse ~~horse-radish~~ horseradish peroxidase (1:10,000 in 1% BSA/TBS plus 0.1% Tween®-20) using the ECL detection system (Amersham).--

Please replace the paragraph beginning at page 47, line 21, with the following redlined paragraph:

--ELISAs were carried out as previously described by Kenny *et al.*, *Infection & Immunity* 65 (1997). For binding/competitive ELISAs, 100 µl of EPEC or *tir* supernatant grown under conditions that induce Tir secretion was added to Immulon 96-well (Dynatech Laboratories, Inc) plates. After blocking with 200 µl 0.1% Tween®-20/PBS, wells were incubated with a) 100 µl of His-T7Int (0.75 µg/ml in PBS) or 2-fold serial dilutions in PBS or b) 100 µl His-T7Int (0.75 µg/ml in PBS) containing 170 µg/ml MBP-Int or 85 µg/ml MBP in addition to 4-fold serial dilutions into PBS containing 0.75 µg/ml His-T7Int. Binding of the His-T7Int fusion was detected with T7 antibodies and visualized spectrophotometrically at absorbance wavelength A490 as previously described by Kenny *et al.*, *Infection & Immunity* 65 (1997).--

Please replace the paragraph beginning at page 48, line 23, with the following redlined paragraph:

--Cloning and sequence analysis of *tir* genes was done as follows: The DNA fragment encoding EHEC *tir* was obtained by PCR from EHEC chromosomal DNA using primers derived from the published sequence of enteropathogenic *E. coli*. Vent DNA

polymerase was used for PCR to amplify chromosomal DNA from EHEC strains. The PCR reaction was carried out for thirty cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, and elongation at 72°C for two minutes. The resulting product was ligated into the commercially available plasmid pBluescript and both strands were sequenced. DNA sequencing was done as follows: The DNA fragment encoding the *tir* genes was amplified by PCR using the primers, and EHEC chromosomal DNA as the DNA template. The resulting blunt end fragment was digested with *SalI* and cloned into the *SalI-SmaI* site of the commercially available plasmid pBluescript-II SK (+). The DNA sequence of EHEC *tir* was determined using the commercially available Taq DyeDeoxy™ kit. Open reading frames were found in the cloned regions and both of these DNA sequences were similar to EPEC *tir*.--

Please replace the paragraph beginning at page 49, line 16, with the following redlined paragraph:

RDEC-1 (SEQ ID NO:5, nucleotide; SEQ ID NO:12, polypeptide) and its *espA* and *espB* mutant strains were inoculated by the orogastric route into young rabbits. Most RDEC-1 was found in the cecum and colon one week postinfection. However, the number of either mutant strain was greatly decreases in these tissues compared to the parent strain. RDEC-1 adhered specifically to the sacculus rotundas (follicle associated epithelium) and bacterial colonization was also observed in the cecum, indicating that the sacculus rotundas in the cecum is an important colonization site for this pathogen. The adherence levels of the *EspA*⁻ and *EspB*⁻ strains to the sacculus rotundas were 70 and 8000 times less than that of parent strain. These results show that the adherence ability and tissue tropism of RDEC-1 are dependent on the two *Esp* secreted proteins. Furthermore, *EspB* appears to play a more critical role than *EspA* in bacterial colonization and pathogenesis. This is the first demonstration that the enteropathogenic *E. coli* secreted proteins, *EspA* and *EspB*, which are involved in triggering of host cell signal transduction pathways, are also needed for colonization and virulence.--